Bidirectional Activity-Dependent Morphological Plasticity in Hippocampal Neurons

Report

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Summary

Dendritic spines on pyramidal neurons receive the vast majority of excitatory input and are considered electrobiochemical processing units, integrating and compartmentalizing synaptic input. Following synaptic plasticity, spines can undergo morphological plasticity, which possibly forms the structural basis for long-term changes in neuronal circuitry. Here, we demonstrate that spines on CA1 pyramidal neurons from organotypic slice cultures show bidirectional activity-dependent morphological plasticity. Using two-photon time-lapse microscopy, we observed that low-frequency stimulation induced NMDA receptor-dependent spine retractions, whereas theta burst stimulation led to the formation of new spines. Moreover, without stimulation the number of spine retractions was on the same order of magnitude as the stimulus-induced spine gain or loss. Finally, we found that the ability of neurons to eliminate spines in an activity-dependent manner decreased with developmental age. Taken together, our data show that hippocampal neurons can undergo bidirectional morphological plasticity; spines are formed and eliminated in an activity-dependent way.

Introduction

The ability to undergo activity-dependent changes in synaptic strength is a hallmark of many neurons in the mammalian central nervous system. Such synaptic plasticity has been intensely studied in the hippocampus, and it is hypothesized that changes in functional connectivity underlie cognitive functions such as learning and memory (Bliss and Collingridge, 1993). While there is ample evidence that synapses in the hippocampus can undergo bidirectional changes of their efficacy (Bliss and Lomo, 1973; Dudek and Bear, 1992; Mulkey and Malenka, 1992; Liu et al., 2004), the physiological and, in particular, the morphological alterations that accompany synaptic long-term potentiation (LTP) and long-term depression (LTD) are only partly understood.

Recent improvements in time-lapse imaging techniques have made it possible to observe changes of dendritic morphology and synaptic function at the level of single synapses in response to plasticity-inducing synaptic activation (reviewed by Yuste and Bonhoeffer, 2001; Emptage et al., 2003). These new technologies have made it possible to test the long-held hypothesis (Cajal, 1911; Hebb, 1949) that functional changes of synapses are accompanied by morphological changes at the level of single spines or synapses. More precisely, it was shown that electrical stimulation resulting in the enhancement of Schaffer collateral/CA1 synapses induces the growth of new postsynaptic spines or filopodia on CA1 pyramidal neurons (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). Recent work shows that spines can also exhibit more subtle modifications, such as changes in their shape after synaptic plasticity (Matsuzaki et al., 2004). These experiments demonstrated that LTP at single synapses is correlated with persistent spine volume increases, a possibility raised many years ago (van Harreveld and Fifkova, 1975).

While several reports demonstrated that strengthening of synapses results in structural changes on dendrites, such as growth of new spines or filopodia, evidence for the opposite effect, namely, the reduction in size or the complete retraction of spines or filopodia in response to LTD-inducing stimulation, has not been reported so far.

Here, we further explored the ability of hippocampal CA1 pyramidal neurons to exhibit electrical stimulation-induced morphological plasticity, by time-lapse imaging of the dendritic structure of green fluorescent protein (GFP)-positive cells with two-photon laser-scanning microscopy (TPLSM). We used two different stimulation protocols, theta burst stimulation (TBS; Larson et al., 1986) and low-frequency stimulation (LFS; Dudek and Bear, 1992; Mulkey and Malenka, 1992), which are classically used to induce LTP or LTD, respectively, at the Schaffer collateral/CA1 synapses. We show that—in keeping with earlier results—an LTP-inducing stimulus leads to the growth of new spines. Importantly, we now also demonstrate that low-frequency stimulation results in morphological plasticity, by a clear and statistically significant loss of spines. In addition to these changes, we also find a constant stimulus-independent loss of spines, which we think can be explained physiologically rather than by "run down" of the slice culture. Finally, we explore the age dependence of activity-dependent morphological plasticity and observe that neurons from 4- to 8-week-old slice cultures show significantly less LFS-induced and stimulus-independent spine loss than younger ones. This suggests that the stability of spines and their resistance to activity-dependent morphological plasticity increases with the developmental age of the tissue.

Our results now provide a link between an LTD-inducing stimulus and spine loss, suggesting that the bidirectional functional synaptic plasticity, LTP and LTD, may be closely associated with bidirectional morphological plasticity.

Results

We used time-lapse TPLSM to image the dendritic morphology of GFP-positive CA1 pyramidal neurons in organotypic hippocampal slices prepared from transgenic

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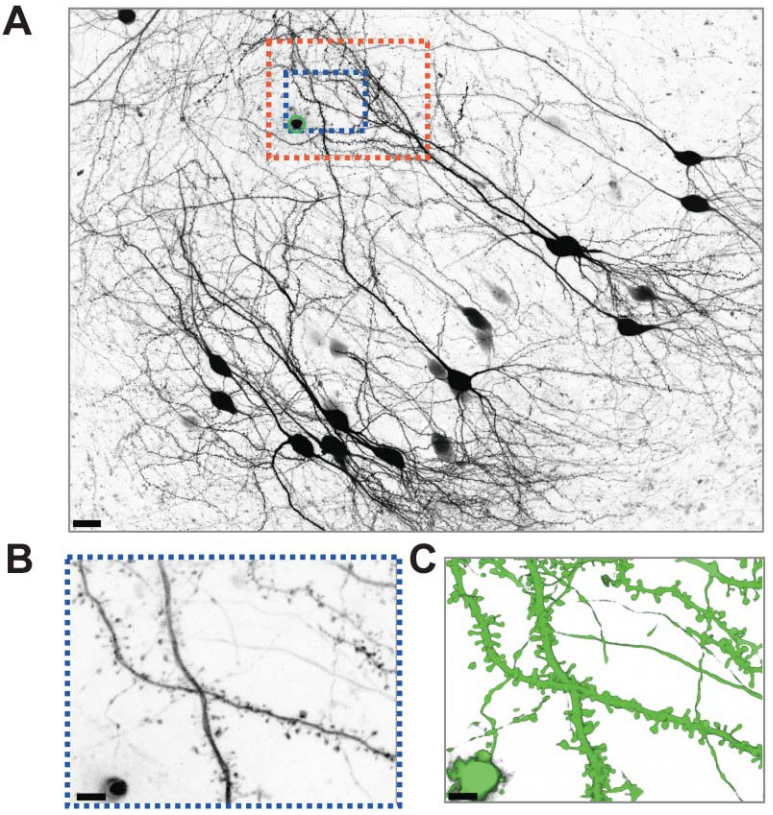


Figure 1. TPLSM Allows Faithful Time-Lapse Imaging of Dendritic Fine Structure

(A) Overview of GFP-positive CA1 pyramidal neurons, the red rectangle outlining a typical field of view containing stretches of dendrites and the fluorescent tip of the electrode (green circle) used for extracellular stimulation. Scale bar, 20 μm. Zooming in on a stretch of dendrite (blue rectangle) either as a raw image (B) or after image processing (C) illustrates the improvement in image quality achieved by Kalman averaging, filtering, and volume rendering. Scale bars in (B) and (C), 5 μm.

mice. Imaging at 30 min intervals, we determined the time course of dendritic spines that appeared and disappeared following electrical stimulation of Schaffer collaterals using two different stimulation protocols. Figure 1A shows fluorescent CA1 pyramidal neurons, the red rectangle indicating a typical region of interest on the apical dendrite, containing the fluorescent tip of the electrode (green circle) used for extracellular stimulation. Figure 1B shows a raw image of a stretch of dendrite, and Figure 1C shows the same image after processing, illustrating the improvement in image quality achieved by Kalman-averaging, filtering, and volume rendering.

Theta Burst Stimulation Induces Spine Growth

Initially, we wanted to confirm that TBS, which is classically associated with N-methyl-D-aspartate (NMDA) receptor-dependent LTP at the Schaffer collateral synapses, is effective at inducing new spines on CA1 pyramidal neurons. Using local field stimulation, we found that dendritic spine growth could indeed be induced by a stimulating electrode positioned in the vicinity of a dendrite. Figures 2A and 2B show representative images from different time series of volume-rendered image stacks before and after TBS stimulation, illustrating the morphological changes that we observed. Figure 2D shows the quantitative analysis of 14 such experiments, with the number of new spines per 100 μm of dendrite plotted against time (solid black circles). As a control, the number of new spines that appeared without any electrical stimulation is plotted as solid red squares.

To test whether TBS led to a significant increase in spine number beyond that attributable to baseline spinogenesis, we compared the total numbers of newly grown spines after TBS and under unstimulated control conditions. Three hours after TBS, the number of new spines per 100 μm of dendrite was 1.01 ± 0.17 spines ($n = 6$) compared to a baseline value of 0.2 ± 0.15 ($n = 8$) ($p = 0.0043$; two-tailed Student's t test). The stimulus-induced spine growth peaks (as indicated by the steepest part of the curve) at about 2 hr after the stimulation and levels out at about 3 hr. Together, these data are in agreement and in support of earlier studies showing that LTP-like stimulation can result in the generation of new spines (Engert and Bonhoeffer, 1999; Toni et al., 1999; Jourdain et al., 2003).

We also quantified the population of spines that first emerged and then disappeared, termed "transient spines." No differences (see Figure 2D, gray symbols and lines) were detected in the rates at which transient spines were created during the unstimulated condition and after TBS. Most transient spines were present only at one data point, indicating that they are relatively short lived.

Theta Burst Stimulation Does Not Induce Spine Retraction

All of these earlier studies focused on the question of whether an LTP-like stimulus leads to the formation of new spines. We also investigated the possibility that TBS, in addition to producing new spines, also leads to the retraction of existing spines. The open symbols in

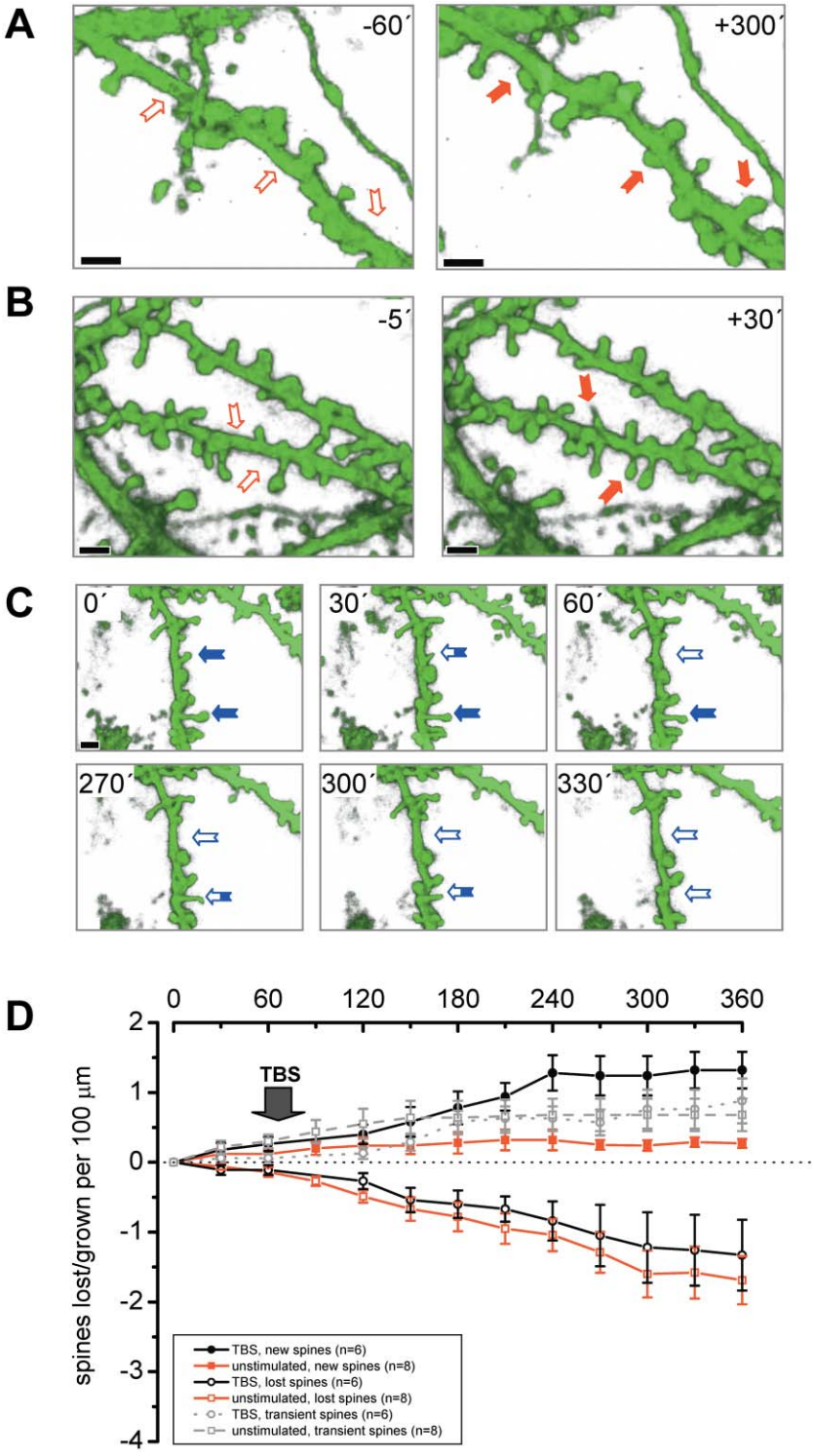


Figure 2. TBS Stimulation Induces New Spine Formation

(A) Stretch of dendrite shown before ($t_0 = -60$ min) and after ($t_1 = +300$ min) TBS stimulation. The open red arrows in the left panel point at sites of later spine growth, indicated by the solid red arrows in the right panel. (B) Another example of TBS-induced spinogenesis ($t_0 = -5$ min; $t_1 = +30$ min). (C) Example of spontaneous (unstimulated) loss of spines, the solid blue arrows pointing at sites of later spine loss as indicated by the open blue arrows in panels showing images taken at later time points. All scale bars, 2 μm. (D) Quantification: number of spines generated or retracted per 100 μm of dendrite for each time point, showing a clear effect of TBS (onset of stimulation indicated by arrow) on the generation of new spines (solid black circles, n = 6) as compared to unstimulated controls (solid red squares, n = 8). In contrast, the number of retracted spines after TBS (open black circles, n = 6) is similar to that of unstimulated controls (open red squares, n = 8). Note that after TBS, the numbers of new and retracted spines are comparable, indicating that there is no net gain in spine number even after TBS. Also indicated in the plots are the time points of the emergence of transient spines after TBS (open gray circles) and during unstimulated conditions (open gray squares). Error bars indicate SEM.

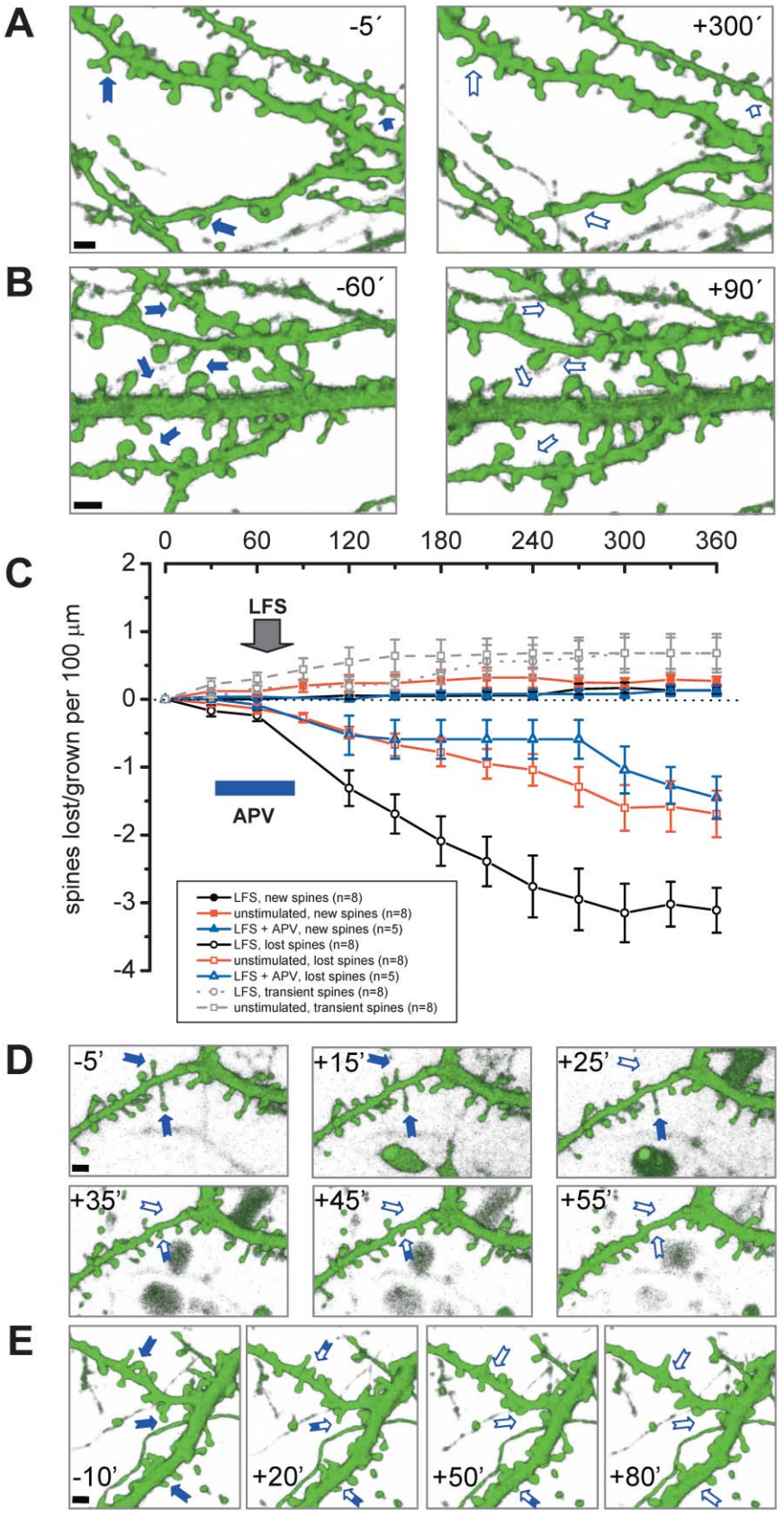

A
-5´
+300´
B
-60´
+90´
C
0
60
120
180
240
300
360
2
1
0
-1
-2
-3
-4
LFS
APV
spines lost/grown per 100 µm
LFS, new spines (n=8)
unstimulated, new spines (n=8)
LFS + APV, new spines (n=5)
LFS, lost spines (n=8)
unstimulated, lost spines (n=8)
LFS + APV, lost spines (n=5)
LFS, transient spines (n=8)
unstimulated, transient spines (n=8)
D
-5'
+15'
+25'
+35'
+45'
+55'
E
-10'
+20'
+50'
+80'

Figure 2D show the number of spine retractions for the stimulated and unstimulated control cases (negative values). The number of spines (per 100 μm of dendrite) that retracted over a 3 hr time window after TBS (0.73 ± 0.29; n = 6) was statistically not distinguishable from the number of the unstimulated control condition (0.9 ± 0.23; n = 8; p = 0.65, two-tailed Student's t test). Figure 2C shows an example of two spines that retracted spontaneously in such an experiment. Interestingly, the number of retractions of the unstimulated control condition is not much smaller than the number of spines grown after TBS (1.01 ± 0.17, n = 6), so that there is hardly any net gain in the number of spines under these conditions (p = 0.42, two-tailed Student's t test).

Low-Frequency Stimulation Induces Spine Retraction

Next, we tested whether a stimulus known to be effective in inducing LTD leads to the retraction of spines on CA1 pyramidal neurons. Apart from using a low-frequency stimulus (900 pulses at 1 Hz), the experiments were identical to the TBS experiments described above. We first confirmed electrophysiologically that local stimulation with LFS indeed caused LTD (data not shown). Representative examples of the morphological effect of this stimulus are shown in Figures 3A and 3B, illustrating that spine retractions can occur in an all-or-none fashion. Furthermore, only some spines retracted, while other spines in the neighborhood remained unaffected for many hours. Blocking NMDA receptors by application of 50 μM D,L-2-amino-5-phosphonovaleric acid (APV) at the time of LFS completely prevented this effect. The open symbols in Figure 3C show the time courses of the spine loss for the conditions of LFS, LFS with 50 μM APV, and without stimulation. The plots for the "no stimulation" (open red squares) and "APV" (open blue triangles) cases show an average decrease in spine number (∼0.25 spines/100 μm/hr), which is very similar to the baseline spine loss that is described in Figure 2D. The data for the LFS show a markedly greater spine loss within 3 hr after the stimulation (2.52 ± 0.43; n = 8), which is significantly different from the control case without stimulation (0.9 ± 0.23; n = 8; p = 0.0074) and APV application with stimulation (0.51 ± 0.29; n = 5; p = 0.0046) over the same time window. Whereas these data quite clearly show that LFS-induced spine retraction is dependent on NMDA receptor activation, they also imply that the baseline spine retractions are independent of NMDA receptor activation.

To be able to characterize in greater detail the kinetics of individual spine retraction events, we repeated the LFS experiments at higher image acquisitions rates (one stack every 10 min as opposed to 30 min). Figures 3D and 3E show representative examples of time series of spine retractions, illustrating that the time it takes for individual spines to retract may range from under 10 min (Figure 3D, upper arrow) to about an hour (Figure 3D, lower arrow).

Low-Frequency Stimulation Does Not Induce Spine Growth

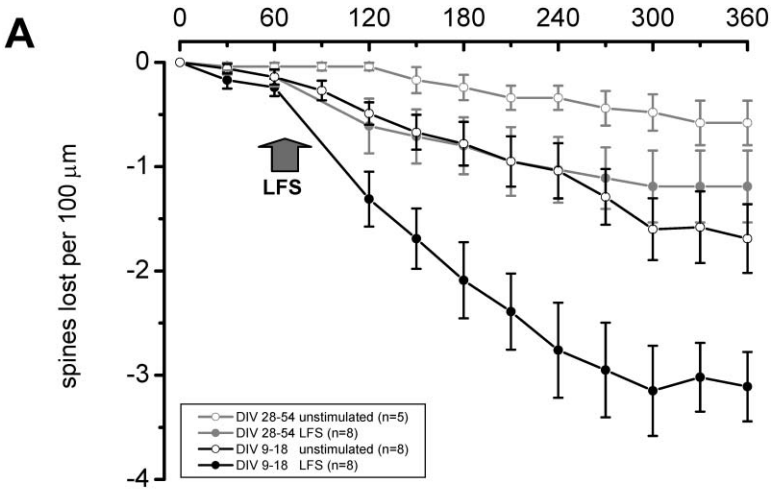
Similar to the reasoning above and in light of the fact that TBS is effective at inducing spine growth, we wanted to find out whether a similar "positive" morphological plasticity is inducible by the LFS protocol. Therefore, we also counted and compared the number of newly grown spines after LFS with the cases of no stimulation and APV application during stimulation. As Figure 3C shows, this protocol did not induce spine growth significantly compared to unstimulated (p = 0.39, two-tailed Student's t test) or APV-treated (p = 0.62, two-tailed Student's t test) conditions. This shows that, opposite to the TBS protocol, the LFS protocol is effective at inducing spine retractions, but ineffective at inducing spine growth on dendrites of CA1 pyramidal neurons. The rate of generation of transient spines (gray symbols and curves) was again indistinguishable between LFS and unstimulated control conditions.

Activity-Dependent Spine Retraction Depends on Developmental Age

We wanted to explore the possibility of whether the activity-dependent morphological plasticity described here is prone to developmental changes. It has been proposed that the maturation of slice cultures continues in vitro and that their age roughly corresponds to the age at which the slice culture was prepared plus the days the tissue has been in culture (De Simoni et al., 2003). We therefore repeated the LFS experiments using older slices cultures that had been cultured for varying times ranging from 28 to 54 days in vitro (DIV). We compared the numbers of spine retractions after LFS between the older cultures with those from the original experiments, (DIV 9–18). The effect of the stimulus on spine retractions is quantified in Figure 4A, showing that the number of spine retractions per 100 μm of dendrite in the older slice cultures is reduced by about 65% compared to the younger ones (0.89 ± 0.26, n = 8 versus 2.52 ± 0.43, n = 8, $t_{240} - t_{60}$, p = 0.0078, two-tailed Student's t test). We also quantified the loss of spines

Figure 3. LFS Stimulation Induces Spine Retraction

(A and B) Representative stretches of dendrites before (−min) and after (+min) LFS. The solid blue arrows on the left panels point at sites of later spine retraction as shown by the open arrows in the right panels. Note that the loss is specific to some spines, sparing other, neighboring spines. All scale bars, 2 μm. (C) Quantification: numbers of new or retracted spines per 100 μm of dendrite, showing that LFS (onset of stimulus indicated by arrow) leads to a marked decrease in the number of spines (open black circles, n = 8) as compared with unstimulated (open red squares, n = 8) or APV-treated controls (open blue triangles, n = 5, duration of APV application indicated by blue bar). Note that the number of retracted spines is comparable between the unstimulated and APV-treated conditions, indicating that the LFS effect on spine retraction requires NMDA-receptor activation and suggesting that baseline spine loss is NMDA receptor independent. LFS does not lead to the formation of new spines, as indicated by the flat curve displaying the number of new spines/100 μm (solid black circles, n = 8), which is indistinguishable from the curves for the unstimulated and APV-treated control conditions. Also plotted is the time course of the emergence of transient spines after LFS (open gray circles) and during unstimulated conditions (open gray squares). Error bars indicate SEM. (D and E) Representative examples of time series of spines in the process of retracting, with the duration ranging from under 10 min to about one hour. The blue-white arrows indicate spines that are in the process of retracting.



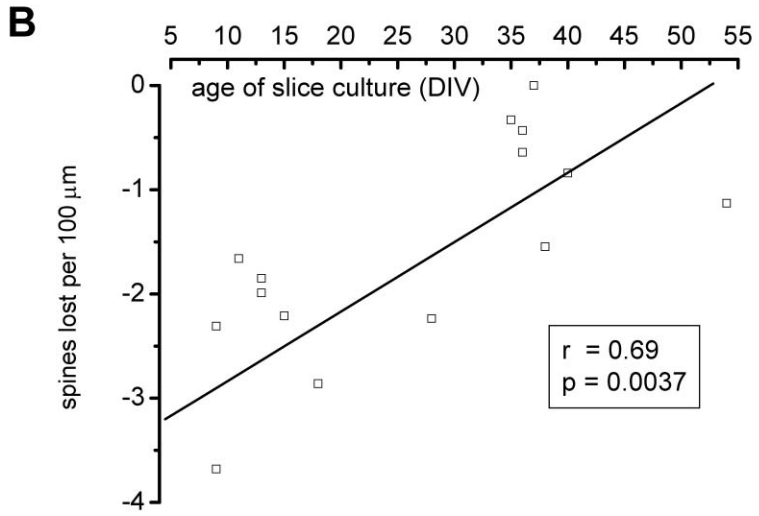


Figure 4. LFS-Induced Spine Retraction and Baseline Spine Loss Depend on Developmental Age

(A) CA1 pyramidal neuron from older slices cultures (DIV 28–54) exhibit a reduced ability to retract spines after LFS (solid gray symbols; time of stimulation indicated by arrow) compared to younger slice cultures (DIV 9–18; solid black symbols). Note that the loss of spines is much less pronounced in the older slice cultures under unstimulated control conditions, indicating that both baseline and LFS-induced spine retractions are significantly more frequent in younger slice cultures. (B) Scatter plot of all LFS experiments, plotting the number of spines retracted 2 hr after LFS (open black squares) against the age of the slice culture. The black line plots the linear regression through the data points, revealing a significant correlation ($r = 0.69$, $p = 0.0037$) between the age and number of spine retractions. Error bars indicate SEM.

under unstimulated conditions in the older slice cultures to be able to assess the degree of baseline loss as a function of the age of the slice culture. Similar to the LFS experiments, the baseline spine loss is markedly reduced by about 65% in the older slice cultures compared to younger ones (0.31 ± 0.13, $n = 5$ versus 0.90 ± 0.23, $n = 8$, $p = 0.049$), indicating that spines are generally more stable in older cultures. Figure 4B shows the entire set of experiments using the LFS protocol, displaying the number of spines lost 3 hr after the stimulation for each experiment against the respective age of the slice culture. The straight line is a linear regression through the data, revealing a significant correlation ($r = 0.69$, $p = 0.0037$). This indicates that the ability of neurons to retract spines in an activity-dependent way indeed decreases with age.

Discussion

We have demonstrated that dendritic spines of hippocampal CA1 pyramidal neurons in organotypic slice cultures exhibit bidirectional activity-dependent morphological plasticity. Using time-lapse TPLSM of GFP-positive neurons, we imaged the dendritic structure before and after extracellular electrical stimulation and assessed its plasticity by counting the numbers of spines that grew or retracted. Our data show that spines can grow de novo or disappear completely depending on the stimulation protocol. Whereas a stimulus that is classically used to induce LTP at the Schaffer collateral synapses (Larson et al., 1986) leads to spine formation, we now find that LFS normally used to induce LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992) induces spine retractions.

The earlier studies on spine formation were careful to state that spinogenesis cannot necessarily be equated with synaptogenesis. Even if the formation of spines leads to new synapses, it is not clear whether these spines actually make contact with the correct presynaptic fibers to support the synaptic enhancement that generated them. The case is similar for spine retraction, where it is also possible (1) that the retracted spines do not eliminate their synapses but that a synaptic connec-

tion is maintained on the dendritic shaft, and (2) that even if synapses are eliminated, it is not clear whether they were part of the connections that underwent LTD. It is therefore an open question whether the activity-dependent loss of spines provides a structural basis for LTD. Since LTD is usually expressed with little delay after LFS, the fact that stimulus-induced spine retractions still occur well after the stimulus may instead point to a homeostatic mechanism that could allow neurons to adjust the synaptic strengths of the remaining synapses to optimal levels within their dynamic range. At this point it remains to be seen whether the functional downregulation of synaptic strength can be attributed—entirely or in part—to the observed loss of spines and potentially synapses. Still, the fact that APV can block LFS-induced LTD at Schaffer collateral/CA1 synapses (Dudek and Bear, 1992; Mulkey and Malenka, 1992), as well as LFS-induced spine loss, suggests that both phenomena are closely related.

A further notable parallel between LFS-induced spine loss and LTD is their age-dependent decline. For LFS-induced LTD it has been reported that the largest effect occurs at P12–20, the ability to undergo LTD is reduced considerably at P31–40, and it completely vanishes from P41 on (Kemp et al., 2000). This time course beautifully parallels our data (Figure 4B), where we find the strongest spine loss during the first 2–3 weeks and spine loss progressively vanishing thereafter.

Interestingly, in young cultures we observed a loss of spines in the absence of any stimulation that clearly outweighs the baseline rate of spine formations, suggesting that the overall spine density should decrease continuously. This could reflect a general run down of the slice culture under our experimental conditions, although we find this unlikely, since single spines are retracted while others remain stable for many hours. Moreover, this baseline loss is much less pronounced in older slice cultures, suggesting a developmental change in the stability of spines and their ability to undergo activity-dependent retraction.

The spine loss might also be explained in the context of a hypothesis originally put forward with respect to synaptic strengthening and weakening (Cooper, 1973) rather than with spine generation and spine loss. Evidently, the creation of new spines (or stronger synapses) has to be counteracted by some homeostatic mechanism to prevent saturation. One way to achieve this is to have a general unspecific loss of spines (or synapse strength; Cooper, 1973; Palm, 1982) to ensure that the average number of spines (or average synapse strength) is maintained. Whether and how such a mechanism is regulated is an interesting question on its own; however, it is beyond the scope of the present paper. It is thus conceivable that the observed baseline spine loss subserves such a homeostatic purpose.

Our study confirms and extends previous work by demonstrating that an LTP-inducing stimulus is accompanied by the growth of new spines on dendrites of CA1 pyramidal neurons. In a departure from the study of Maletic-Savatic et al. (1999), who reported the outgrowth of filopodial processes after prolonged high-frequency stimulation, we found that TBS led to the growth of dendritic structures resembling bona fide spines. We only very rarely observed the appearance of new filopodia. This is in line with a report from our own laboratory, which showed that "pairing" of pre- and postsynaptic activity under a local superfusion paradigm causes the growth of new spines after the induction of synaptic plasticity (Engert and Bonhoeffer, 1999). However, the number of new spines after LTP-inducing stimulation reported in this earlier study is higher than what we report here. A potential explanation for this discrepancy may be provided by the different stimulation paradigms used in the two studies. On one hand, the local superfusion approach (Engert and Bonhoeffer, 1999), by which all nonsuperfused parts of the dendrite are silenced, is quite different from local stimulation which leaves the whole dendrite in its normal state of activity. On the other hand, the difference between TBS (used in the present study) and "pairing" (used in the previous study) might also account for at least part of the numerical difference in newly generated spines.

Moreover, the spine growth that we observe here and its magnitude agrees well with a recent study by Jourdain et al. (2003), who report the emergence of approximately one new spine per 100 μm length of dendrite under comparable stimulation conditions. Taken together, these findings lend strong support to the hypothesis that functional synaptic plasticity should be reflected in alterations of neuronal morphology (Cajal, 1911; Hebb, 1949) and open the prospect of studying activity-dependent synapse turnover in real time in an in vitro system. Whether the synaptic activation-induced growth of new spines can be equated with the formation of new functional synapses has not been determined yet, but other studies (Trachtenberg et al., 2002) have shown that newly generated spines—albeit not by an activity-dependent paradigm—entail the formation of ultrastructurally mature synapses.

Our study shows that spines cannot only be formed de novo but that they can also be retracted in an activity-dependent way. This suggests that the functional plasticity of LTP and LTD is mirrored by bidirectional morphological plasticity. It is tempting to speculate that these morphological changes might be used to stabilize functional changes to make them more permanent. It should be kept in mind, however, that there are other potential functions for these morphological changes, and further experiments will be needed to determine their role. The fact that both "positive" and "negative" morphological changes occur in concert with their functional counterpart suggest that they have an important role in shaping neural connections.

Experimental Procedures

Organotypic Hippocampal Slice Cultures and Recording Solutions

Hippocampal slices (300 μm thick) from postnatal day 5–7 transgenic mice (Thy-1 promoter, GFP-M mouse line, courtesy of J. Sanes, Washington University, St. Louis, MO) were prepared, embedded in a plasma clot on glass coverslips, and incubated for up to 8 weeks in a roller incubator at 35°C, according to the Gähwiler method (Gähwiler, 1981). The age of the slice cultures for the experiments is expressed in days in vitro after the preparation and indicated in the text. For the experiments, cultures were transferred into a recording chamber, where they were continuously perfused with carbogenated (95% O_2, 5% CO_2) ACSF containing NaCl, 126 mM; KCl, 2.5 mM; $CaCl_2$, 2.8 mM; $MgCl_2$, 0.5 mM; glucose, 10 mM;

NaH_2PO_4, 1.25 mM; $NaHCO_3$, 26 mM; glycine, 0.05 mM; and pyruvate, 1 mM. The temperature was maintained at 35°C, and the pH was 7.4. In some control experiments, 50 μM APV was bath applied for 45 min (30 min before and throughout LFS).

Electrophysiology

Patch pipettes were used for electrical stimulation. They were filled with 3 M NaCl and 10 mM of the fluorescent dye calcein immobilized in agar. A chlorided silver wire was used to pass brief current pulses (0.2 ms) of 15–30 μA from a stimulus isolator (WPI, Berlin, Germany) through the patch pipette. The stimulus protocols used for LTP or LTD were as follows: LTP, theta burst stimulation, consisting of five trains (200 ms intertrain interval) of six pulses delivered at 100 Hz, repeated five times every 10 s; LTD, low-frequency stimulation, consisting of 900 pulses delivered at 1 Hz. The tip of the electrode was positioned in the vicinity of the stretch of apical dendrite that was selected for time-lapse imaging. The minimal distance between the tip of the electrode and the dendrite was kept between 10 and 20 μm. In separate experiments, we verified that this arrangement of stimulus electrode and stimulation strength reliably produced subthreshold synaptic potentials that were not contaminated by direct stimulation of voltage-gated conductances in the postsynaptic cell.

Microscopy

Time-lapse two-photon laser-scanning microscopy was used to image over time the dendritic morphology of GFP-positive CA1 pyramidal neurons. The red excitation light ($\lambda = 840$ nm) from a 5 W Mira-Verdi laser system (Coherent, Santa Clara, CA) was routed through a Fluoview 200 scanner (Olympus, Hamburg, Germany), a suitable dichroic mirror (LOT Oriel, Darmstadt, Germany) and a 40×, 1.2 NA water-immersion objective (Zeiss, Oberkochen, Germany) mounted on an inverted IX70 microscope (Olympus). The power of the excitation light could be adjusted continuously by an acousto-optical modulator (Polytec, Waldbronn, Germany), and its average value at the objective was set to 10–20 mW. The fluorescence was detected by an external photomultiplier tube (R6357, Hamamatsu, Herrsching, Germany). Image acquisition and online analysis was performed by the Fluoview software (Olympus), and image stacks were saved to disk for offline analysis. The nominal image resolution was 115 nm/pixel in x-y. A piezo-electrical actuator (Physik Instrumente, Karlsruhe, Germany) was used to move the objective in the z axis ($\Delta z = 0.4$ μm). Images were Kalman averaged over three frames. One stack was acquired every 30 min, with three stacks recorded before the stimulation and ten stacks afterward. For the high time resolution experiments, different acquisition rates were used, the highest being one image stack per 3 min. A reference image was taken at the start of the experiment to facilitate alignment and to keep sample drift over the 6 hr of the experiment at a minimum.

Image Analysis

4D (x, y, z, t) image stacks were processed and analyzed using the Imaris 4 software (Bitplane, Zürich, Switzerland). Individual stacks were spatially filtered by an edge-preserving algorithm, rescaled, and baseline subtracted. To facilitate overview and for display in the figures, the 3D stacks were volume rendered as 2D images using the blending projection view in Imaris. All image analysis, however, was done by visual inspection of the individual image sections as well as the rendered 2D projections of the image stacks.

A total of 16,201 μm of dendrite was analyzed (395 ± 220 μm per experiment; mean ± SD). Data obtained from a total of 41 slices cultures were included in the analysis. Changes in dendritic morphology were divided into three groups. (1) *New spines*, which were absent in the first image stack(s), emerged at a later time point, and remained present throughout the rest of the experiment. (2) *Transient spines*, which were not present in the first image stack(s), appeared later, and then disappeared again without reappearing for the duration of the experiment. Typically, transient spines were observed during a single time point. In a few instances, they persisted over multiple time points. The time point of their emergence is plotted in the graphs. Note that the fact that transient spines are usually seen in only one time point means that we must be underestimating the true number of transient spines. (3) *Retracted spines*, which were present in the first image stack(s), disappeared at a later time point and did not reappear for the duration of the experiment. These three categories were sufficient to fully describe the "behavior" of the spines that we observed under these experimental conditions. No attempt was made to quantify possible shape or size changes of spines. Rather, we focused on those changes that were all-or-none and that were well within the resolution of TPLSM. To confirm the spine count statistics, 10 out of the 43 experiments were selected at random and subjected to a recount by an operator blind to the experimental conditions. This analysis yielded qualitatively the same results.

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